Hepatic glutathione and nitric oxide are critical for hepatic insulin-sensitizing substance action

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Guarino, Maria P., Ricardo A. Afonso, Nuno Raimundo, João F. Raposo, and M. Paula Macedo. Hepatic glutathione and nitric oxide are critical for hepatic insulin-sensitizing substance action. Am J Physiol Gastrointest Liver Physiol 284: G588–G594, 2003. First published December 4, 2002; 10.1152/ajpgi.00423.2002.—We tested the hypothesis that hepatic nitric oxide (NO) and glutathione (GSH) are involved in the synthesis of a putative hormone referred to as hepatic insulin-sensitizing substance HISS. Insulin action was assessed in Wistar rats using the rapid insulin sensitivity test (RIST). Blockade of hepatic NO synthesis with Nω-nitro-L-arginine methyl ester (L-NAME, 1.0 mg/kg intraportal) decreased insulin sensitivity by 45.1 ± 2.1% compared with control (from 287.3 ± 18.1 to 155.3 ± 10.1 mg glucose/kg, \( P < 0.05 \)). Insulin sensitivity was restored to 321.7 ± 44.7 mg glucose/kg after administration of an NO donor, intraportal SIN-1 (5 mg/kg), which promotes GSH nitrosation, but not after intraportal sodium nitroprusside (20 nmol·kg\(^{-1}\)·min\(^{-1}\), which does not nitrosate GSH. We depleted hepatic GSH using the GSH synthesis inhibitor L-buthionine-[S,R]-sulfoximine (BSO, 2 mmol/kg body wt ip for 20 days), which reduced insulin sensitivity by 39.1%. Insulin sensitivity after L-NAME was not significantly different between BSO- and sham-treated animals. SIN-1 did not reverse the insulin resistance induced by L-NAME in the BSO-treated group. These results support our hypothesis that NO and GSH are essential for insulin action.

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resistance induced by l-NAME in hepatic GSH-depleted rats was assessed. Our results were consistent with the hypothesis that HISS synthesis in the liver requires NO as well as GSH.

METHODS

Presurgical Protocols

Animals were treated according to the Laboratory Animal Care Guidelines of the European Union (86/609/CEE) and the National Institutes of Health. Male Wistar rats (8–9 wk old) were housed one per cage, with a 12:12-h light-dark cycle under controlled temperature. Rats were fed standard rat chow (Panlab A04, Charles River) ad libitum, except for the day before surgery, when the rats were fasted overnight and then allowed access to food for 1 h. Experiments began between 9 and 10 AM. Rats were anesthetized with pentobarbital sodium (65 mg/kg ip), and anesthesia was maintained throughout the experiment by continuous infusion of pentobarbital sodium (1.0 mg/ml, 1.0 ml·100 g body wt$^{-1}$·h$^{-1}$) through a cannula inserted in the internal jugular vein. The temperature was maintained at 37.0 ± 0.5°C by means of a heating pad (Homeothermic Blanket Control Unit 50-7061, Harvard Apparatus) and monitored with a rectal probe thermometer. Rats were heparinized with 100 IU/kg heparin.

GSH Depletion

Five-week-old male Wistar rats received BSO (BSO group, n = 5) or saline (sham group, n = 6). BSO was dissolved in saline and administered at 2 mmol·kg$^{-1}$·day$^{-1}$ ip for 20 days between 10 and 12 AM following previously described protocols (10). The sham group received intraperitoneal saline for the same period of time. On day 20, the day before surgery, the rats were fasted overnight and then allowed access to food for 1 h. Rats were anesthetized, and the presurgical procedure described above was followed.

Surgical Preparation

The trachea was cannulated (polyethylene fusing PE-240; Becton Dickinson) to allow spontaneous respiration. The arteriovenous (a-v) loop previously described (15) was primed with a saline-heparin solution (200 IU/ml). The a-v loop forms a vascular shunt connected by cannulation (PE-50; Becton Dickinson) of the carotid artery with the arterial side of the loop and cannulation of the left jugular vein with the venous side of the loop. Arterial blood samples (25 μl) were obtained by puncture of the loop sleeve. Arterial blood pressure was monitored by briefly clamping the venous outlet of the loop. The patency of flow in the shunt was also monitored by recording pressure from the nonocluded loop. Insulin, glucose, and anesthetic were administered intravenously by puncturing (infusion line PE50 with a cut 23 gauge needle at the end) the loop on the venous side. The portal vein was cannulated with a 24-gauge iv intravenous catheter (Jelco, Johnson & Johnson Medical) after laparotomy.

Rats were allowed to stabilize from the surgical intervention for 50 min before any procedures were carried out. Arterial blood samples were collected every 5 min after stabilization, and glucose concentrations were immediately analyzed by the oxidase method with a glucose analyzer (1500 YSI SPORT, Yellow Springs Instruments) until three successive stable glucose concentrations were obtained. The mean of these three values is referred to as the basal glucose level.

RIST

The RIST, which is a modified euglycemic clamp reproducible for four consecutive tests in the same animal, has previously been described (15).

Insulin infusion was started using an infusion pump (Perfusor, Braun) to administer the dose of insulin (50 mU/kg iv) over 5 min. After 1 min of insulin infusion, arterial blood glucose was measured, and glucose infusion (d-glucose-saline, 100 mg/ml iv) was started at a rate of 5 mg·kg$^{-1}$·min$^{-1}$. According to arterial glucose concentrations measured at 2-min intervals, the infusion rate of the glucose pump was readjusted to maintain euglycemia. When no further glucose infusion was required, usually within 35 min, the test period was concluded. The amount of glucose infused after insulin administration quantifies insulin sensitivity and is referred to as the RIST index (mg glucose/kg) (15).

HISS Quantification

The RIST index is composed of two components: the HISS-dependent component and the HISS-independent component. In this study, the HISS-independent component, or insulin action per se, is obtained by inhibition of hepatic NO release through direct administration of l-NAME into the portal vein. The HISS-dependent component of insulin action is calculated by subtracting the RIST index obtained after NOS blockade from the control RIST index, as previously reported (27, 35).

Experimental Protocols

Effect of intraportal administration of the NOS antagonist l-NAME on insulin sensitivity. After the control RIST was performed, l-NAME (1 mg/kg) was infused intraportally over 5 min. A stable basal arterial glucose concentration was determined, and a new RIST was performed, 30 min after l-NAME infusion. After restabilization, consecutive RISTs were performed at 90, 140, and 190 min after l-NAME infusion to evaluate the duration of action of the l-NAME dose (n = 5).

Effect of intravenous vs. intraportal administration of the NO donor SNP on l-NAME-induced insulin resistance. The RIST index was determined before and 60 min after intraportal infusion of l-NAME (1 mg/kg, 5-min bolus). SNP (20 nmol·kg$^{-1}$·min$^{-1}$) was administered intraportally (n = 6) or intravenously (n = 5) 90 min after l-NAME. After a basal glucose level was established, a new RIST was performed.

Effect of intraportal administration of the NO donor SIN-1 on l-NAME-induced insulin resistance. The RIST index was determined before and 60 min after intraportal infusion of l-NAME (1 mg/kg, 5-min bolus). SIN-1 (5 mg/kg, 5-min bolus) was infused intraportally (n = 6) 90 min after l-NAME infusion, and the RIST was repeated.

Effect of GSH depletion on insulin sensitivity and determination of the HISS-dependent component of insulin action. A control RIST was performed as described in the BSO (2 mmol/kg body wt ip, 20 days, n = 5) and sham (intraperitoneal saline, n = 6) groups to evaluate insulin sensitivity. Thereafter, the RIST index was determined 60 min after intraportal infusion of l-NAME (1 mg/kg, 5-min bolus) in the BSO or sham group to evaluate HISS action.

Effect of the NO donor SIN-1 on l-NAME-induced insulin resistance in GSH-depleted rats. SIN-1 (5 mg/kg, 5-min bolus) was infused intraportally 90 min after l-NAME, and the RIST was repeated in the BSO (n = 5) and sham (n = 6) groups. Rats were allowed to stabilize between each RIST.
blood pressure increased after L-NAME infusion from 119.3 ± 4.0 to 141.4 ± 3.2 mmHg at 20 min (P < 0.01) and 131.7 ± 6.7 mmHg after 190 min.

Effect of Intravenous vs. Intraportal Administration of the NO Donor SNP on L-NAME-Induced Insulin Resistance

Neither intraportal (142.5 ± 15.8 mg glucose/kg, n = 6) nor intravenous (110.5 ± 34.1 mg glucose/kg, n = 5) infusion of SNP (20 nmol·kg⁻¹·min⁻¹) affected the RIST index after L-NAME (Figs. 1 and 2, respectively). The mean arterial blood pressure increased after L-NAME infusion from 125.3 ± 8.1 to 136.0 ± 6.8 mmHg, and it remained stable at 131.7 ± 6.7 mmHg for ~2 h. The mean arterial blood pressure decreased after intraportal SNP (from 124.2 ± 4.2 to 95.0 ± 2.6 mmHg, P < 0.01) and after intravenous SNP (from 123.0 ± 8.0 to 88.0 ± 3.7 mmHg, P < 0.01), but it remained constant during the RIST.

Effect of Intraportal Administration of the NO Donor SIN-1 on L-NAME-Induced Insulin Resistance

The control RIST index of 271.3 ± 37.6 mg glucose/kg was significantly reduced to 152.2 ± 21.3 mg glucose/kg (P < 0.01) after intraportal infusion of L-NAME (1 mg/kg, n = 5). Intraportal administration of SIN-1 (5 mg/kg) completely reversed the inhibition caused by L-NAME (321.7 ± 44.7 mg glucose/kg; Fig. 3). After L-NAME infusion, the mean arterial blood pressure increased from 115.8 ± 8.5 to 127.5 ± 5.3 mmHg and remained at this level during the RIST. The mean arterial blood pressure decreased (from 123.3 ± 5.4 to 80.8 ± 6.5 mmHg, P < 0.001) after SIN-1 administration but remained constant during the RIST.

Effect of GSH Depletion on Insulin Sensitivity and Determination of the HISS-Dependent Component of Insulin Action

The RIST index was significantly lower in the BSO group (158.4 ± 12.2 mg glucose/kg, n = 5; Fig. 4) than in the sham group (260.2 ± 15.6 mg glucose/kg, n = 6,
Therefore, GSH depletion with BSO reduced insulin sensitivity by 39.1%. Intraportal infusion of L-NAME (1 mg/kg) decreased the RIST index in the BSO and sham groups. In the BSO group, the RIST index after L-NAME was 109.0 ± 9.1 mg glucose/kg, corresponding to a change from control of 30.6 ± 4.4% (Fig. 4); in the sham group, the RIST index was 121.2 ± 12.8 mg glucose/kg, corresponding to a change from control of 52.3 ± 5.8% (P < 0.05; Fig. 5). HISS action, quantified by subtracting the post-L-NAME RIST from the control RIST, was 138.9 ± 22.8 mg glucose/kg for the sham group and only 49.3 ± 8.6 mg glucose/kg for the BSO group (P < 0.01), which corresponds to a 64.4% reduction of HISS action after BSO treatment.

**Effect of the NO Donor SIN-1 on L-NAME-Induced Insulin Resistance in BSO-Treated Rats**

In the BSO group, intraportal SIN-1 (5 mg/kg) did not reverse the decrease in insulin sensitivity caused by L-NAME (77.8 ± 12.4 mg glucose/kg; Fig. 4). However, in the sham group, SIN-1 completely restored insulin sensitivity (258.1 ± 18.5 mg glucose/kg, P < 0.001; Fig. 5).

**Hepatic GSH Determination**

GSH concentration was decreased in the BSO group, as expected. The GSH levels were 5.9 ± 0.4 μmol/g fresh liver in the sham group and 3.0 ± 0.4 μmol/g fresh liver in the BSO group, which corresponds to a decrease of 49.2% (P < 0.001; Fig. 6).

**DISCUSSION**

A novel neurohumoral regulatory mechanism for insulin action was recently reported (13). According to this mechanism, insulin action at the skeletal muscle consists of two components: one is mediated through hepatic NO, and the other is independent of NO production in the liver (28). The hepatic NO-dependent component is responsible for the release of a putative hormone referred to as HISS, which accounts for ~55% of the whole body insulin action (14). Recent studies (14) have demonstrated that HISS release is impaired in the fasted state and maximal in the immediate postprandial state. It has also been suggested that lack of HISS release after feeding leads to insulin resistance typical of type 2 diabetes (13). Hepatic GSH levels are depleted during fasting (31), and serum GSH...
levels are decreased in type 2 diabetes (17, 33, 34), suggesting the involvement of GSH in HISS release, as well as in insulin action. Our findings confirm the hypothesis that hepatic GSH, together with NO, is required for full HISS-dependent insulin action.

In the fed state, insulin resistance induced by NOS antagonism can be restored by providing NO to the liver (7, 27). According to Sadri and Lautt (27), intraportal administration of L-NAME at 1 mg/kg significantly reduced the response to insulin, whereas administration of the same dose intravenously did not cause a significant decrease in the insulin response. Insulin sensitivity was restored after administration of intraportal, but not intravenous, SIN-1. These experiments support the hypothesis that hepatic NO mediates the HISS-dependent component of insulin action (13, 27).

In the present work, we studied the ability of different NO donors to reverse HISS-dependent insulin resistance. Two NO donors with distinct chemistries were used. SIN-1 decomposes nonenzymatically to yield NO and superoxide (O2•−) (5). Recent data suggest that simultaneous production of NO and O2•− is an intrinsic activity of NOS and that the enzyme does not catalyze production of free NO unless high concentrations of superoxide dismutase (SOD) are present (18). According to these findings, SIN-1, as an NO/O2•− provider, might be regarded as the donor that better mimics NOS activity (30). Schrammel et al. (30) showed that NO/O2•− rapidly reacts with GSH to produce an intermediate with biological activity, S-nitrosoglutathione (GSNO). The formation of GSNO from NO/O2•− is clearly different from the nitrosation of GSH by peroxynitrite (ONOO−); the reaction of GSH with NO/O2•− is 20-fold more efficient than the reaction of GSH with ONOO− (19). In addition, ONOO− formation is partially outcompeted by the rapid reaction of NO/O2•− with GSH, which seems to occur preferentially, even in the presence of SOD (18).

The other NO donor used in the present study, SNP, releases NO promptly and spontaneously, without O2•− formation (5). Free NO does not nitrosate thiols at significant rates (9); thus SNP has a low capacity to nitrosate hepatic GSH. Therefore, the main difference between the two NO donors is their ability to nitrosate thiols: SIN-1 promotes GSNO formation, and SNP does not (9, 11, 18).

In the light of the facts presented, we suggest that intraportal SIN-1 was able to reverse L-NAME-induced insulin resistance because it mimics hepatic NOS activity by releasing NO/O2•− simultaneously and, likely, inducing GSNO synthesis in the liver, which, we hypothesize, is important for HISS secretion. Intrahepatic administration of SIN-1 does not reverse HISS inhibition caused by NOS antagonism, confirming that the drug is acting through the liver (27). Neither intraportal nor intravenous administration of SNP reversed insulin resistance induced by L-NAME, probably because of inability of free NO to nitrosate thiols (9), which appears to be essential for triggering HISS synthesis. Another alternative hypothesis to explain why SNP did not restore insulin sensitivity is that NO might have been scavenged by hemoglobin (2). However, administration of SNP, intravenously and intraportally, promoted a decrease in mean arterial blood pressure, showing that NO was not being inactivated by hemoglobin. Although other investigators suggest that insulin resistance induced by NOS blockade is secondary to a reduction in skeletal muscle perfusion and, consequently, a reduction in the delivery of insulin and glucose to its target tissues (1), in our testing conditions we observed that the administration of the NO donor SNP did not improve insulin sensitivity, despite its notorious vasodilatory effects. Furthermore, if the insulin resistance observed after NOS blockade was secondary to inhibition of the dilatory responses to insulin in skeletal muscle, intravenous administration of SNP or SIN-1 should have produced more pronounced effects on insulin action than the intraportally administered dose. Neither we nor Sadri and Lautt (27) observed any effect of intravenous NO donor infusion in restoring impaired insulin action after L-NAME administration. Our results agree with our previously described hypothesis that insulin resistance in skeletal muscle caused by NOS blockade is related to a hepatic effect of NO, rather than its vascular effects.

Several lines of evidence suggest that increased oxidative stress may play a role in peripheral insulin resistance (4, 20, 22). Subjecting muscle and fat cells to oxidative stress has been shown to result in a dramatic decrease of insulin-stimulated glucose transport (12, 25, 32) and glycogen synthesis (3). In adipocytes, H2O2 impairs various metabolic effects of insulin, including the stimulation of glucose uptake activity, through alterations in the expression and translocation capacity of the glucose transporter GLUT-4 (10, 25). It was also reported that oxidative stress has an inhibitory effect on tyrosine phosphorylation of the insulin receptor β-subunit (8).

Decreased GSH levels were found in adipocytes exposed to an H2O2-generating system (26) and also in blood and tissues of diabetic rats (17, 33, 34), which raises the possibility that decreased GSH impairs insulin action through enhancement of oxidative stress. Nevertheless, diminished glucose tolerance was observed in GSH-depleted rats, although in vitro insulin responsiveness in skeletal muscle and adipocytes was preserved (10). Khamaisi et al. (10) describe the intriguing observation that GSH depletion by BSO was progressively associated with abnormal glucose tolerance tests, which could not be attributed to impaired insulin secretion or insulin action in skeletal muscle or adipose tissue. According to these authors, GSH levels in the muscle were reduced by 14 ± 1% in skeletal muscle; nevertheless, the muscle responsiveness to insulin, assessed ex vivo by measuring 2-deoxyglucose uptake in the absence and presence of insulin, was not significantly different in BSO-treated and control animals, although in vivo impaired glucose tolerance was observed (10).

Other studies (21, 24) report beneficial metabolic effects of antioxidants when administered to diabetic subjects. De Mattia et al. (4) state that GSH infusion

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increases total glucose uptake in type 2 diabetes patients.

The exposure to oxidative stress conditions is characterized by decreased GSH-to-GSSG ratio, decreased GLUT-4 protein and mRNA expression, and generation of lipid peroxidation products (3, 10, 25, 32). However, these oxidative stress markers were not altered in BSO-treated compared with control rats by administration of BSO at 2 mmol·kg⁻¹·day⁻¹ (10). With regard to the possible effects of oxidative stress induced by GSH depletion on insulin action, administration of BSO at 2 mmol·kg⁻¹·day⁻¹ allowed us to assess the isolated effect of GSH depletion without mimicking the complex reactions associated with oxidative stress (10, 12). GSH depletion by BSO did not significantly affect basal or insulin-stimulated glucose uptake in adipocyte and skeletal muscle cell lines, in contrast with H₂O₂ exposure, which significantly impaired glucose uptake activity in the same conditions (10). This supports the hypothesis that the effects of GSH depletion by BSO on insulin sensitivity are not secondary to oxidative stress.

Our hypothesis is that decreased hepatic GSH levels promote an HISS-dependent impairment of insulin responsiveness in skeletal muscle. In BSO-treated rats, GSH levels decreased by 49.8% and insulin sensitivity decreased by 39.1%. After i-NAMe infusion, the RIST index was similar in the BSO and sham groups, indicating that HISS-independent insulin action was normal and that GSH depletion affected HISS action only. Moreover, SIN-1 administration completely restored insulin sensitivity in the sham but not in the BSO group. We, therefore, suggest that insulin resistance observed in BSO-treated rats is due to hepatic GSH depletion and subsequent decreased ability to synthesize GSNO, which we hypothesize is involved in HISS release.

We have shown that in the fasted state, when HISS release is suppressed, intraportal SIN-1 administration is not able to restore HISS-dependent insulin action (unpublished observations), probably because hepatic GSH levels are reduced in the fasted state (31).

Our findings support the hypothesis that NO and GSH are essential in HISS synthesis/release. Additional studies are required to evaluate the role of GSH/NO in animal models of insulin resistance having in mind further pharmacological manipulations to increase HISS-dependent insulin sensitivity.

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